BRIEF COMMUNICATIONS

Direct Effects of Cholinergic Stimulation on Ventricular Automaticity in Guinea Pig Myocardium

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SUMMARY. The purpose of these experiments was to determine whether muscarinic cholinergic agonists exerted a negative chronotropic effect in the absence of endogenous norepinephrine in isolated guinea pig ventricular myocardial strips. The chronotropic response to physostigmine (10^{-6} M) in control, reserpinpretreated animals, and in the presence of increased norepinephrine release induced by superfusion of tyramine (10^{-5} M), was studied. The control rates in the control, reserpine-pretreated, and tyramine-treated groups were 106 ± 40, 93 ± 31, and 109 ± 28/min, respectively. Propranolol (10^{-6} M) produced a 23% slowing in rate in control animals and an 8% slowing in reserpine pretreated animals (P < 0.01), suggesting basal secretion of norepinephrine. Tyramine (10^{-5} M) produced a 28% increase in rate in control animals (P < 0.05) and tyramine (10^{-4} M) produced no increase in reserpine-pretreated animals. Physostigmine produced similar negative chronotropic response in control, reserpine-pretreated, and tyramine-treated groups of 45, 49, and 28%, respectively. Physostigmine produced no change in measured Purkinje fiber action potential characteristics, except for a decreased rate of spontaneous diastolic depolarization. Our results demonstrate that physostigmine slows the spontaneous rate in control, reserpine-pretreated and tyramine-treated groups, indicating that muscarinic cholinergic agonists exert a direct negative chronotropic effect at postjunctional cell surface receptors, independent of the presence or level of adrenergic tone. (Circ Res 52: 105-110, 1983)

Vagal stimulation and muscarinic cholinergic stimulation have been shown to alter ventricular function significantly (DeGeest et al., 1965; Daggett et al., 1967; Randall et al., 1968; Wildenthal et al., 1969), exerting negative inotropic (Hollenberg et al., 1965) as well as negative chronotropic effects (Bailey et al., 1972; Tse et al., 1976) effects in the mammalian ventricle. Vagal stimulation or muscarinic cholinergic agonists applied in the presence of simultaneous adrenergic stimulation results in a magnification of these negative inotropic and chronotropic effects (Levy et al., 1966; Dempsey and Cooper, 1969; Levy and Zieske, 1969; Levy, 1971; George et al., 1973; Watanabe and Besch, 1975; Bailey et al., 1979).

These previous studies show accentuated effects of cholinergic agonists in the presence of increased adrenergic tone or were not designed specifically to evaluate the possibility that the absence of endogenous norepinephrine release might modify the effect of the cholinergic agonists. The presence of ventricular tissue norepinephrine stores which are released from postganglionic sympathetic terminals (Burnstock and Holman, 1962) might increase adrenergic tone and accentuate the effects of muscarinic cholinergic agonists. One might argue, then, that muscarinic cholinergic effects on the ventricle are indirect, dependent on underlying basal adrenergic tone. Therefore, the purpose of this study was to examine the chronotropic effects of muscarinic cholinergic activation when endogenous norepinephrine was depleted, to determine whether muscarinic cholinergic agonists had a direct effect on guinea pig ventricular electrical function, independent of simultaneous adrenergic stimulation.

Methods

Guinea pigs of either sex which weighed 350-500 g were injected intraperitoneally with heparin sulfate (500 U) 30 minutes prior to use. Each guinea pig was stunned with a blow to the head, after which the heart was rapidly removed and placed in cool oxygenated Tyrode's solution. Strips of the left ventricular free wall which measured approximately 1 cm² were transected to a thickness of approximately 4 mm. Each strip included the endocardium and endocardial Purkinje fiber network. The strips were affixed to the floor of a wax-bottomed Lucite muscle chamber that was superfused constantly with Tyrode's solution, gassed with 95% O₂-5% CO₂, and maintained at a temperature of 37 ± 0.5°C. The composition of the Tyrode's solution was (in mM): Na⁺, 141; K⁺, 4.0; Cl⁻, 127; Ca²⁺, 2.0; HCO₃⁻, 22.0; H₂PO₄⁻, 0.9; Mg²⁺, 0.5; and glucose, 5.5.

A surface electrogram was recorded from the endocardial surface of each ventricular strip. The electrogram was displayed on a Tektronix 5100 series oscilloscope and photographed with a Tektronix C-59 Polaroid oscilloscope camera. In addition, the experiments were recorded with a Hewlett-Packard 3946 tape recorder. An electrogram-triggered tachometer was used to record the spontaneous ven-
The tachometer produced a linear ramp, the height of which was inversely proportional to the spontaneous ventricular rate. This method enabled us to observe the cycle length of every spontaneous depolarization throughout the course of an experiment. The tachometer was calibrated by triggering the ramp at known basic cycle lengths and measuring the height of the ramp. In addition, in several experiments from each experimental group, conventional microelectrode techniques (Draper and Weidmann, 1951) were used to record transmembrane action potentials of endocardial Purkinje fibers that demonstrated spontaneous diastolic depolarization. Transmembrane action potentials from ventricular muscle tissue were recorded in selected experiments. Action potential data are reported only from experiments in which a single microelectrode impalement was maintained throughout the course of an experiment.

The ventricular strips were superfused in the muscle chamber for 20 minutes after a stable automatic rate was achieved prior to any experimental intervention. Only ventricular strips with a stable spontaneous rate were used for further study. Drugs were administered by continuous infusion into the muscle chamber, using a Harvard constant flow infusion pump. The spontaneous ventricular rate was determined from the tachometer recording after a steady state rate with each pharmacological intervention was achieved. Effects of physostigmine sulfate, tyramine hydrochloride, dl-propranolol, and atropine sulfate were studied in ventricular strips with a stable spontaneous rate to increase during superfusion with tyramine (10^{-5} M). The effects of physostigmine (10^{-6} M), an acetylcholinesterase inhibitor, was used to provide indirect muscarinic cholinergic stimulation as in previous studies from this laboratory (Bailey et al., 1979; Mirro et al., 1979; Mirro et al., 1980). The reserpine-pretreated animals were administered reserpine 3 mg/kg, intraperitoneally, 24 hours prior to study. This dosage has previously been shown to deplete tissue norepinephrine stores measured spectrophotofluorimetrically (Dahlstrom and Haggendal, 1966). Adequate reserpine pretreatment was documented in all experiments by failure of the spontaneous ventricular rate to increase during superfusion with tyramine (10^{-4} M). The effects of each pharmacological intervention were recorded in control and reserpine-pretreated animals.

Data were analyzed by one-way analysis of variance with repeated measures. When the analysis of variance was significant, comparisons with control values were made by the Bonferroni method. Paired t-tests were used when only one measurement was compared to the control value. Comparisons between control and reserpine-pretreated groups were made by Student’s unpaired t-test (Wallenstein et al., 1980).

Drugs used include atropine sulfate, tyramine hydrochloride, and physostigmine sulfate purchased from Sigma Chemical Company. Reserpine was purchased from CIBA and dl-propranolol hydrochloride was from Ayerst Chemical Company. All solutions were prepared fresh prior to superfusion.

## Results

The effects of superfusion with physostigmine were studied in left ventricular strips obtained from control and reserpine-pretreated animals. The effects of physostigmine superfusion in animals not pretreated with reserpine were also studied during simultaneous superfusion of tyramine (10^{-5} M). The control rates were 106 ± 40, 93 ± 31, and 109 ± 28/min, respectively, in the three groups. There was no statistically significant difference among the control rates in the groups by nonpaired t-test analysis. In the third group, tyramine (10^{-5} M) was superfused prior to the addition of physostigmine, and produced an increase in the spontaneous rate from 87 ± 42 to 109 ± 28/min (P < 0.05, n = 7). Tyramine superfusion then was continued throughout physostigmine treatment in the third group.

Transmembrane Purkinje fiber action potentials were recorded from several preparations in each experimental group. Purkinje fiber action potentials from the control group had an amplitude of 108 ± 13 mV, take-off potential of —77 ± 8 mV, maximum diastolic potential of —80 ± 7 mV, and spontaneous diastolic depolarization with a slope of 9 ± 3 mV/sec. There was no statistical difference among action potential characteristics in the three groups. Ventricular muscle transmembrane action potentials recorded during experiments in each experimental group exhibited a mean resting potential of —85 ± 3 mV, a mean amplitude of 117 ± 4 mV, and exhibited no spontaneous diastolic depolarization.

The effects of physostigmine (10^{-6} M), dl-propranolol (10^{-6} M), and tyramine (10^{-5} M) on the spontaneous ventricular rate in the control guinea pigs are summarized in Table 1. Physostigmine, a cholinesterase inhibitor, provided indirect muscarinic cholinergic stimulation and resulted in a 45% slowing of the ventricular rate. The effect of physostigmine was completely reversed by atropine (10^{-6} M). In the control animals, dl-propranolol resulted in a statistically significant slowing of the ventricular rate (23%). Tyramine, which causes release of endogenous norepinephrine stores from sympathetic nerve terminals, resulted in a 25% increase in the spontaneous ventricular rate.

To assess whether the negative chronotropic effect of physostigmine in control animals was dependent on adrenergic stimulation from endogenously released norepinephrine, we performed similar studies in reserpine-pretreated guinea pigs. Representative electrogram-triggered tachometer recordings demonstrating the effects of physostigmine (10^{-6} M), dl-propranolol (10^{-6} M), and tyramine (10^{-5} M) are shown in Figure 1, and effects on the spontaneous ventricular rates are summarized in Table 2. Physostigmine superfusion produced a 49% decrease in rate. Propranolol produced no change in rate in reserpine-pretreated animals. The change in rate produced by propranolol in control and reserpine-pretreated groups was statistically different by Student’s unpaired t-test (P < 0.01). Tyramine superfusion resulted in no increase in the spontaneous ventricular rate in the reserpine-pretreated group, indicating that reserpine had adequately depleted norepinephrine stores. An additional seven reserpine-pretreated ani-
mals were studied with simultaneous superfusion of propranolol (10^-6 M) and physostigmine. The addition of propranolol did not alter the negative chronotropic response of physostigmine was similar in reserpine-pretreated animals compared to reserpine-pretreated animals with simultaneous superfusion of propranolol, 49 to 42% slowing, respectively. 

In control animals, evidence for spontaneous release of norepinephrine was suggested by a slightly (though not statistically) increased control rate compared to reserpine-pretreated animals, 106 ± 40 vs. 93 ± 31/min, respectively. Additionally, propranolol produced a greater negative chronotropic response in control compared to reserpine-pretreated animals, 23% to 8%, respectively (P < 0.01). To test further whether the negative chronotropic response to muscarinic cholinergic agonists was altered by the release of endogenous norepinephrine, we studied the effects of physostigmine (10^-6 M) on ventricular strips simultaneously superfused with tyramine (10^-3 M). As noted, superfusion with tyramine produced a 23% increase (P < 0.05) in rate prior to superfusion with physostigmine. Physostigmine superfusion produced a 28% slowing of the spontaneous ventricular rate in tyramine-treated ventricular strips (Table 3).

Superfusion with physostigmine produced no change in measured action potential characteristics, except for a decrease in rate of diastolic depolarization. Representative microelectrode recordings depict the response to tyramine, physostigmine, and atropine in reserpine-pretreated (Fig. 2) and tyramine-treated (Fig. 3) animals, respectively. These analog records demonstrate that the effects of physostigmine were antagonized by atropine (10^-6 M). The diastolic effect of physostigmine on the rate of spontaneous depolarization in control, reserpine-pretreated, and tyramine-treated groups are summarized in Table 4. There was no difference in the effect of physostigmine on the rate of diastolic depolarization among the groups.

**Discussion**

Vagal stimulation has previously been shown to alter mammalian ventricular inotropy and chronotropacy. Muscarinic cholinergic agonists exert a direct negative inotropic effect on ventricular myocardium (Hollenberg et al., 1965). When vagal stimulation or muscarinic cholinergic agonists are applied in the presence of simultaneous adrenergic stimulation, the negative inotropic effects are magnified (Levy et al., 1966; Dempsey and Cooper, 1969; Levy and Zieske, 1969; Levy, 1971; Watanabe and Besch, 1975). Similarly, muscarinic agonists previously have been shown to exhibit effects on His-Purkinje automaticity that is species dependent. In the canine, acetylcholine decreases the spontaneous rate of discharge (Eliakim et al., 1961) and depresses spontaneous diastolic depolarization in the specialized conduction system (Bailey et al., 1972; Tse et al., 1976; Gadsby et al., 1978). In the sheep, acetylcholine produces an increase in the slope of diastolic depolarization and at lower K+ concentrations (3.4 or 2.7 mM/liter^-1) results in spontaneous activity (Carmeliet and Ramon, 1980).

Effects of muscarinic cholinergic agonists on action potential characteristics also are species dependent. In canine cardiac Purkinje fibers, acetylcholine either produces no change in action potential duration (Bailey et al., 1979) or shortens action potential duration (Gadsby et al., 1978). In sheep, acetylcholine increases action potential duration (Carmeliet and Ramon, 1980; Lipsius and Gibbons, 1980).

### Table 1

<table>
<thead>
<tr>
<th>Drug</th>
<th>n</th>
<th>Control</th>
<th>10 min</th>
<th>20 min</th>
<th>30 min</th>
</tr>
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<tbody>
<tr>
<td>Physostigmine</td>
<td>12</td>
<td>97 ± 36</td>
<td>79 ± 35</td>
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<td>Propranolol</td>
<td>7</td>
<td>141 ± 30</td>
<td>128 ± 38</td>
<td>118 ± 40</td>
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<tr>
<td>Tyramine</td>
<td>7</td>
<td>87 ± 42</td>
<td>109 ± 29</td>
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</table>

Spontaneous ventricular discharge rate per minute, mean ± SD, at 10, 20, and 30 minutes of superfusion.

* P < 0.05; † P < 0.01; ‡ P < 0.001 compared to control.
plateau and shortening of the action potential (Ochi and Hino, 1978; Hino and Ochi, 1979).

Muscarinic cholinergic agonists also antagonize catecholamine effects in ventricular tissue. Acetylcholine antagonizes isoproterenol-induced action potential shortening in canine cardiac Purkinje fibers (Bailey et al., 1979) and inhibits the catecholamine-induced slow response (Bailey et al., 1979). Whether the effect of muscarinic cholinergic agonists on action potential characteristics is entirely dependent on simultaneous adrenergic stimulation is unclear. In sheep Purkinje fibers, the prolongation of the action potential and increase in the rate of diastolic depolarization produced by acetylcholine are not blocked by propranolol or phentolamine (Carmeliet and Ramon, 1980).

Evidence exists that there is resting secretion of norepinephrine from postganglionic sympathetic nerves (Burnstock and Holman, 1962), although this spontaneous release of norepinephrine is reduced when the preganglionic sympathetic fibers are cut (Hertting et al., 1962). The hypothesis that this baseline secretion of norepinephrine might modulate the chronotropic response to muscarinic cholinergic stimulation was tested in the present experiments, and muscarinic stimulation was found to have a similar chronotropic response in control, tyramine-treated, and norepinephrine-depleted guinea pig subendocardial ventricular tissue.

The evidence also suggests that there is secretion of norepinephrine from postganglionic sympathetic nerves in the isolated ventricular strips. However, the effect of this secretion on the automatic rate of dis-

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Control</th>
<th>10 min</th>
<th>20 min</th>
<th>30 min</th>
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<tr>
<td>Physostigmine (10⁻⁶ M)</td>
<td>12</td>
<td>94 ± 53</td>
<td>73 ± 40*</td>
<td>55 ± 42*</td>
<td>48 ± 45*</td>
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<tr>
<td>Propranolol (10⁻⁶ M)</td>
<td>5</td>
<td>98 ± 26</td>
<td>99 ± 23</td>
<td>92 ± 23</td>
<td>90 ± 21</td>
</tr>
<tr>
<td>Tyramine (10⁻⁵ M)</td>
<td>19</td>
<td>94 ± 44</td>
<td>92 ± 45</td>
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Spontaneous ventricular discharge rate per minute, mean ± SD, at 10, 20, and 30 minutes of superfusion.

* P < 0.05 compared to control.

### Table 3

<table>
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<tr>
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<th>Physo + Tyramine</th>
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<tr>
<td>Control</td>
<td>Physostigmine (10⁻⁸ M)</td>
</tr>
<tr>
<td></td>
<td>10 min</td>
</tr>
<tr>
<td>87 ± 42</td>
<td>109 ± 28*</td>
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</table>

Spontaneous ventricular discharge rate per minute, mean ± SD; Physo = Physostigmine (10⁻⁸ M).

* P < 0.05 compared to control; † P < 0.001 compared to tyramine alone, n = 7.
response in all groups tested, and the magnitude of this response was similar. This effect was not reduced in reserpine-pretreated animals and was not enhanced in ventricular strips superfused with tyramine, suggesting that, in guinea pig left ventricular strips, the chronotropic response to muscarinic cholinergic agonists is independent of endogenous norepinephrine secretion. Rather, our data indicate a direct effect of muscarinic cholinergic agonists at the myocardial cell surface receptor. Microelectrode impalements of both myocardial cells and Purkinje fibers suggested that, in isolated guinea pig ventricular strips, the automatic focus arose from subendocardial Purkinje fibers. Transmembrane recordings from Purkinje fibers showed spontaneous diastolic depolarization, and occasional cells demonstrated a smooth transition from diastolic depolarization to the upstroke of the action potential. Recordings from ventricular myocardial cells demonstrated no diastolic depolarization. Although our microelectrode recordings are suggestive of an automatic focus within the Purkinje network, it is impossible to be certain a recording from any cell represents the true pacemaker cell.

Muscarinic cholinergic agonists have direct myocardial effects, but also are inhibitors of norepinephrine release from sympathetic nerve terminals (Löffelholz and Muscholl, 1969; Langer, 1974; Vanhoutte and Levy, 1980). In reserpine-pretreated animals, depleted of norepinephrine stores, this prejunctional inhibitory effect on norepinephrine release would be eliminated as a variable in provoking a negative chronotropic response. Muscarinic cholinergic agonists inhibit release of norepinephrine from sympathetic nerve terminals elicited by nerve stimulation or by potassium, whereas this prejunctional inhibition by muscarinic cholinergic agonists is not involved in the regulation of norepinephrine release elicited by tyramine (Löffelholz and Muscholl, 1969; Langer, 1974). Therefore, the prejunctional role of muscarinic inhibition cannot be directly discerned from these experiments. However, our results support the viewpoint that the chronotropic response to muscarinic stimulation is mediated via a direct myocardial effect at the postjunctional muscarinic cholinergic receptor and not secondary to prejunctional inhibition of norepinephrine release.

Postjunctional cholinergic modulation of adrenergic effects on ventricular myocardial function has previously been demonstrated in the mammalian ventricle, but the direct cholinergic effects on spontaneous rate of discharge, independent of adrenergic stimulation, were not previously well established. Acetylcholine attenuates the positive inotropic effects of isoproterenol in isolated perfused guinea pig hearts (Watanabe and Besch, 1975), and muscarinic cholinergic agonists inhibit catecholamine-induced activation of glycogen phosphorylase in isolated hearts (Gardner and Allen, 1977; Watanabe et al., 1978). However, direct (adrenergic independent) effects of muscarinic agonists have previously been shown only in mammalian atrial and avian atrial and ventricular myocardium (Biegon and Pappano, 1980). Our study demonstrates an equipotent negative chronotropic response to muscarinic cholinergic stimulation in control, tyramine-treated, and norepinephrine-depleted guinea pig ventricular tissue. In addition, our results suggest that this negative chronotropic response is due to a direct postjunctional cellular effect on the endocardial Purkinje fibers, independent of the presence of discernable adrenergic tone.

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**Table 4**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Before Phys</th>
<th>After Phys</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (7)</td>
<td>9.1 ± 3.5</td>
<td>5.6 ± 1.8*</td>
</tr>
<tr>
<td>Reserpine-pretreated (4)</td>
<td>9.4 ± 4.2</td>
<td>5.9 ± 4.6†</td>
</tr>
<tr>
<td>Tyramine-treated (4)</td>
<td>13.5 ± 6.1</td>
<td>10.2 ± 4.2*</td>
</tr>
</tbody>
</table>

Rate of diastolic depolarization in mV/sec, mean ± so; Physo = Physostigmine $10^{-6}$ M.

* $P < 0.05$; † $P < 0.01$ compared to control.
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INDEX TERMS: Physostigmine • Muscarinic cholinergic stimulation • Reserpine • Guinea pig ventricle • Ventricular automaticity
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